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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of:

K. KINO, ET AL.

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For:Method for producing amino acids by fermentation

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Group: 1651

EXAMINER: Irene Marx

DECLARATION PURSUANT TO 37 C. F. R. 1.132

Sir:

I, Tetsuya Abe, of 4-17-9, Morino, Machida-shi, Tokyo 194-0022 Japan do hereby declare as follows:

I graduated from Division of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, in March, 1992, and got a master's degree from Kyoto University in March, 1994. Since April, 1994, I have been employed by Kyowa Hakko Kogyo Co. Ltd. I was assigned to Technical Research Laboratories of the company in July, 1994, and was engaged in research and development of efficient

fermentation production system.

I am one of the co-inventors of the invention described and claimed in the application and have a full knowledge of the present invention and cited references.

I conducted the following experiment to show that a microorganism belonging to Escherichia coli which have an enhanced productivity of L-histidine can be obtained by imparting an increased resistance to primaquine disodium salt to an L-histidine producing strain.

Experiment

<Methods>

To obtain a strain which produces L-histidine, Escherichia coli W3110(ATCC27325) was subjected to mutagenesis in an aqueous solution containing N-methyl-N'-nitro-N-nitrosoguanidine (NTG, 0.2 mg/ml) at 30°C for 30 minutes. Mutagenized cells were spread on an agar plate of the minimal medium(2g/l glucose, 6g/l disodium hydrogen phosphate, 3g/l potassium dihydrogen phosphate, 0.25g/l magnesium sulfate, 1g/l ammonium chloride, 0.5g/l sodium chloride, 1 g/l adenine) containing 1 g/l beta-(2-thiazolyl)-DL-alanine. After 2 to 4 days-incubation at 30°C, about 200 colonies appeared and 60 larger colonies were picked up onto LB plates (10g/l Tryptone, 5g/l yeast extract, 10g/l sodium chloride, 16g/l agar).

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Each of the picked up strains and Escherichia coli W3110 was inoculated into 4 ml of an LB medium (10g/l Tryptone, 5g/l yeast extract, 10g/l sodium chloride) in a test tube, and cultured at 30°C for 12 hours under aerobic condition with shaking. After completion of the culturing, 0.5 ml of each of the resulting culture was transferred into test tubes containing 5 ml of a generation culture medium (20g/l glucose, 10g/l corn steep liquor, 24g/l ammonium sulfate, 4g/l potassium dihydrogen phosphate, 0.15g/l magnesium sulfate, 10 mg/l thiamine chloride salt, 10 mg/l calcium pantothenate, 30g/l calcium carbonate, pH 6.5), and cultured at 30°C for 36 hours under aerobic condition with shaking. After culturing, the amount of accumulated L-histidine in the culture was assayed by high-performance liquid chromatography.

Among the cultured and picked up-strains, Escherichia coli TZA-51(hereinafter referred to as TZA-51 strain) was selected as a strain which produces L-histidine in the culture.

TZA-51 strain was subjected to mutagenesis in an aqueous solution containing 0.2 mg/ml NTG at 30°C for 30 minutes. Mutagenized cells were spread on an agar plate of the minimal medium (2g/l glucose, 6g/l disodium hydrogen phosphate, 3g/l potassium dihydrogen phosphate, 0.25g/l magnesium sulfate, 1g/l ammonium chloride, 0.5g/l sodium chloride, 1g/l adenine) containing 400 mg/l primaquine disodium salt at which concentration TZA-51 strain could not grow. After 2 to 6 days-incubation at 30°C, about 100 colonies appeared

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and 30 larger colonies were picked up onto LB plates (10g/l Tryptone, 5g/l yeast extract, 10g/l sodium chloride, 1.6% agar).

Each of the picked up strains, Escherichia coli W3110, and TZA-51 strain was inoculated into 4 ml of an LB medium (10g/l Tryptone, 5g/l yeast extract, 10g/l sodium chloride) in a test tube, and cultured at 30°C for 12 hours under aerobic condition with shaking. After completion of the culturing, 0.5 ml of each of the resulting cultures was transferred into a test tubes containing 5 ml of a generation culture medium (20g/l glucose, 10g/l corn steep liquor, 24g/l ammonium sulfate, 4g/l potassium dihydrogen phosphate, 0.15g/l magnesium sulfate, 10 mg/l thiamine chloride salt, 10 mg/l calcium pantothenate, 30g/l calcium carbonate, pH 6.5), and cultured at 30°C for 36 hours under aerobic condition with shaking. After culturing, the amount of accumulated L-histidine in the culture was assayed by high-performance liquid chromatography.

The L-histidine productivity of Escherichia coli W3110, TZA-51 strain and four strains among 30 primaquine disodium salt resistant-strains (TPQ-1 strain, TPQ-3 strain, TPQ-8 strain and TPQ-11 strain, respectively) is shown in Table 1.

<Results>

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Table 1

Bacterial strains	L-histidine (g/l)
W3110	0
TZA-51	0.16
TPQ-1	0.28
TPQ-3	0.25
TPQ-8	0.28
TPQ-11	0.22

As shown in Table 1, TPQ-1 strain, TPQ-3 strain, TPQ-8 strain and TPQ-11 strain having more resistance to primaquine disodium salt than TZA-1 strain produced more L-histidine than TZA-51 strain.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: This day of January 25, 2005.

Tetsuya Abe

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